Plant Biotechnology 23, 215–218 (2006)

Gene Note

Isolation and expression analysis of phosphate transporter genes from *Eucalyptus camaldulensis*

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Received January 17, 2006; accepted January 26, 2006 (Edited by T. Hashimoto)

Abstract Five distinct genomic DNA fragments (*EcPT1*, *EcPT2*, *EcPT3*, *EcPT4* and *EcPT5*) encoding phosphate transporters were isolated from *Eucalyptus camaldulensis*. *EcPT2* and *EcPT3* were exclusively expressed in the root, but were not enhanced by phosphate deprivation. The transcript level of *EcPT2* was much higher than that of *EcPT3*. The *EcPT2* is present as a single copy gene in *Eucalyptus* genome, and phylogenetic analysis and deduced amino acid sequence revealed that *EcPT2* can be classified into the Pht1 family, a high-affinity phosphate transporter. These results suggest that *EcPT2* functions in phosphate transport in root tissues not only under low-phosphate conditions as with typical Pht1 family members, but also under high-phosphate conditions.

Key words: *Eucalyptus*, phosphate transporter, Pht1 family.

Phosphorus (P) is one of the most important nutrients for plant growth and, since it is deficient in most soils, limits plant growth in most natural ecosystems (Bieleski 1973). In agricultural systems, a large input of phosphate fertilizer is required since P is acquired by plants in the form of inorganic phosphate (Pi). Plants use a series of strategies to enhance Pi acquisition from soils, including secretion of several molecules (protons, organic acids, phosphatases and nucleases), changes in root morphology and up-regulation of Pi transporters (Poirier and Bucher 2002; Rausch and Bucher 2002). Of these strategies, it is likely that Pi transporters in roots play a major role in Pi uptake from the soil. Understanding the molecular mechanisms of Pi uptake is thought to be an important step in gene manipulation of high-yield plants in Pi-deficient soils.

Although Pi transport has been extensively investigated in various plants, including agriculturally important crops, little is known with regard to industrially important wood species. Recently, with the increasing demand for renewable energy, the importance of fiber and chemical materials from wood has been growing rapidly. Breeding of trees that can grow in nutrionally poor soil will expand the plantation area, increase wood production and reduce pressure of exploitation of native forests.

Eucalyptus is important as a raw material for industrial pulp and paper making. In this study, as a first step in understanding the molecular mechanism of Pi uptake in *Eucalyptus*, we focused on the Pi transporter genes of this species.

Pi transporter genes were screened from the *E. camaldulensis* genomic library. A cDNA fragment encoding a putative Pi transporter derived from the *Eucalyptus* EST database constructed by Oji Paper Co. Ltd. (Japan) was used as a probe for plaque hybridization. Five partial genomic DNA sequences with high homology to the Pi transporter gene were isolated. These genes were designated *EcPT1* (identical to the EST sequence used as probe), *EcPT2*, *EcPT3*, *EcPT4* and *EcPT5*. Gene accession numbers are AB242816, AB242817, AB242818, AB242819 and AB242820, respectively. Each of these partial DNA sequences shares around 80% homology.

Expression of these five genes was analyzed by northern blot analysis. *EcPT2* and *EcPT3* mRNA were expressed in a root-specific manner and were not induced by Pi-deficiency (Figure 1). Moreover, *EcPT2* mRNA accumulation was much higher than that of *EcPT3*. Expression of *EcPT1*, *EcPT4* and *EcPT5* was not observed in the current experiment. Further, although *EcPT1* was observed in the EST database, the transcript of *EcPT1* was not detected. This might have been due to the low expression of *EcPT1* in the experimental

Abbreviations: MES, 2-(*N*-Morpholino) ethanesulfonic; P, phosphorus; Pi, inorganic phosphate; PT, phosphate translocator; TM, transmembrane.
This article can be found at http://www.jspcmb.jp/

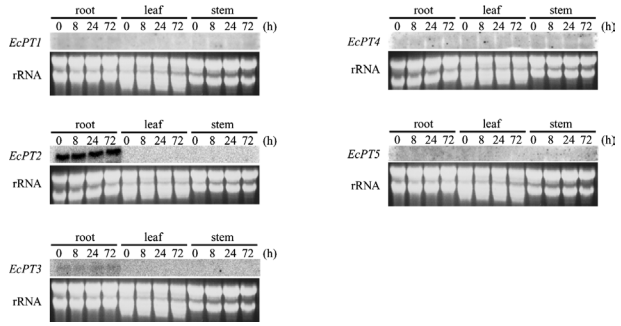

Figure 1. Expression analysis of the *EcPT* genes in *E. camaldulensis*. *Eucalyptus* plants with the same genetic background were used throughout this study. Plantlets were grown in 1/4 strength Gamborg's B5 medium solution supplemented with 0.5 g l^{-1} 2-(*N*-Morpholino) ethanesulfonic (MES) (pH 5.6) for about 1 month in a greenhouse. To confirm the response to Pi deficiency, medium containing 250 μM NaH_2PO_4 was replaced with Pi-deficient medium containing 250 μM NaCl 0 h, 8 h, 24 h and 72 h before sampling. Total RNA was isolated from the leaves, stems and roots as described by Suzuki et al. (2003). Aliquots of 15 μg of RNA were electrophoretically separated on formaldehyde agarose gel and blotted onto a nylon membrane. Hybridization was carried out according to a standard procedure. Details of the sequence information of the probes and primers used for amplification are available from the authors on request.

conditions used in this study.

We therefore focused on *EcPT2* since the expression level of this gene in the roots was extremely high. *EcPT2* has one intron within its coding region (Figure 2A). The deduced amino acid sequence of *EcPT2* consists of 535 amino acids with an apparent molecular mass of 58.4 kDa. Genomic Southern blot analysis showed the presence of a single *EcPT2* gene in *E. camaldulensis* (Figure 2B).

In vascular plants, genes encoding Pi transporters and Pi translocators (PT) play a crucial role in Pi transport systems. Recently, Pi transporters were functionally and structurally classified into three families, namely, Pht1, Pht2 and Pht3 (Rausch and Bucher 2002). Phylogenetic analysis of EcPT2 with *Arabidopsis* Pi transporters/translocators demonstrated a close relationship between EcPT2 and the Pht1 family (Figure 2C). Kinetic data for Pht1 members showed that they were high-affinity Pi transporters and involved in acquisition of Pi by the roots from low external concentrations in the soil (Daram et al. 1998; Mitsukawa et al. 1997). The TMAP program (http://bioinfo.limbo.ifm.liu.se/tmap/) predicted the presence of 12 transmembrane domains in EcPT2 and a central hydrophilic domain separating six N-terminal domains from six C-terminal domains (Figure 2D). This domain structure is common among other high-affinity Pi transporters of higher plants, yeast and fungi (Ming et al. 2005; Harrison et al. 2002; Paszkowski et al. 2002; Rausch et al. 2001; Muchhal et al. 1996; Harrison et al. 1995; Bun-Ya et al. 1991). The phosphorylation sites for protein kinase C and casein kinase II and the site for potential *N*-glycosylation are conserved in high-affinity Pi transporters (Ming et al. 2005; Kai et al. 2002; Muchhal et al. 1996) and are also present in EcPT2 at positions 238-240 (T-A-R), 505-508 (S-L-E-E) and 419-422 (N-A-T-T), respectively.

To date, many genes encoding a high-affinity Pi transporter homologous to yeast *PHO84* have been identified in plants, helping elucidate Pi uptake mechanisms (Poirier and Bucher 2002; Rausch and Bucher 2002). Most are expressed predominantly in root tissues and are strongly induced by Pi deprivation. In *Arabidopsis*, all *Pht1 genes*, except *Pht1;6*, are expressed either exclusively or predominantly in the roots, and are induced under conditions of Pi deprivation (Mudge et al. 2002). In rice, 10 of the 13 Pi transporters are expressed in the roots (Paszkowski et al. 2002). Root-specificity and Pi-deficiency inducible expression are typical features of *Pht1* family genes across various plant species (Ming et al. 2005; Schunmann et al. 2004; Chiou et al. 2001; Daram et al. 1998).

In this study, five putative Pi transporter genes were isolated from the genomic library of *E. camaldulensis*. Of these, only two, designated *EcPT2* and *EcPT3*, were expressed constitutively in a root-specific and Pi-independent manner in our experimental conditions. Nucleotide sequencing of the *EcPT2* implied that this gene encodes a high-affinity Pi transporter, suggesting that EcPT2 functions as a Pi transport both in low and high Pi conditions. Isolation of a full set of *Pht1* genes and detailed expression studies of *EcPT2* are currently in progress with the aim of further understanding the Pi transport mechanism in *Eucalyptus*.

Figure 2. Properties of *EcPT2*. (A) Nucleic and deduced amino acid sequences of *EcPT2*. The deduced amino acid of the ORF is shown under the nucleic acid sequence. An intron (underlined) was presumed using a splicing site prediction program (NetGene2 Server; http:/www.cbs.dtu.dk/service/NetGene2/). This splicing site was also confirmed by sequencing of *EcPT2* cDNA amplified from *Eucalyptus* root RNA by RT-PCR using gene-specific primers. (B) Southern blot analysis of *EcPT2*. Genomic DNA was extracted from *Eucalyptus* leaf as described by Wagner et al. (1987) with minor modifications. Aliquots of 15 μg of genomic DNA were digested with the indicated restriction enzymes, electrophoretically separated on agarose gel and blotted onto a nylon membrane. DIG-labeled (Roche, Germany) *EcPT2* DNA (+1086 to +1609 relative to the translational start site with no restriction enzyme site used for the genomic DNA digestion) was used as a probe. Hybridization was carried out according to a standard procedure. (C) The phylogenetic tree of EcPT2 and *Arabidopsis* Pi transporter/translocator proteins. Amino acid sequences were aligned using ClustalW and the tree was constructed with TreeView software. (D) Amino acid sequences of EcPT2 and its related Pi transporter proteins. The deduced amino acid sequence of proteins EcPT2, Pht1;1, Pht1;4 and Pht1;6 were aligned. The predicted transmembrane (TM) domains of EcPT2 are indicated by a dotted line. The phosphorylation sites of protein kinase C (*) and casein kinase II (#) and the site of potential *N*-glycosylation (+) are indicated.

A

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   1 atggctaaag aaaatcttgg ggtgctaaat gcactgatg tggccaagac acaatggtac
     M A K E  N L G  V L N  A L D V  A K T  Q W Y
  61 catttcaccg ccatcatcat tgccggcatg gggttcttca ccgacgcgta tgatctcttt
     H F T A  I I I  A G M  G F F T  D A Y  D L F
 121 tgcgtttccc tcgtcacgaa gctcctcggt cgcatatact actatgaggg taaggataag
     C V S L  V T K  L L G  R I Y Y  Y E G  K D K
 181 cctggctcgc tccctccgaa cgtggctgcc gctgtcaacg gcgtcgcctt ctgcggtact
     P G S L  P P N  V A A  A V N G  V A F  C G T
 241 ctagccggcc agctcttctt cggctggctt ggggacaaat taggtcggaa gcgtgtctat
     L A G Q  L F F  G W L  G D K L  G R K  R V Y
 301 ggtttaaccc tcattattat gatcatctgc tccgtcggct ccggcttgtc ctttggggat
     G L T L  L I M  I I C  S V G S  G L S  F G D
 361 tcaccgaata gtgttatagc aaccctatgc ttcttccgat tctggctcgg gtttgggatc
     S P N S  V I A  T L C  F F R F  W L G  F G I
 421 ggaggggatt acccactctc cgccaccatc atgtcggagt acgcgaacaa gaagactcgt
     G G D Y  P L S  A T I  M S E Y  A N K  K T R
 481 ggggcattca tcgctgcagt gtttgcgatg caagggttta ggattttggg gggtgggatc
     G A F I  A A V  F A M  Q G F G  I L G  G G I
 541 gtcgctttga ttgtctcctc agcattcgat cataagttta aggccccgcc ttacgaagtc
     V A L I  V S S  A F D  H K F K  A P P  Y E V
 601 aatccagtgg gctcgaccgt tcctcaagcc gattacgtgt ggcgtatcat tgtaatgctc
     N P V G  S T V  P Q A  D Y V W  R I I  V M L
 661 ggtgcattac ccgcggccct tacttattat tatagaatga aaatgcctga aactgctcgt
     G A L P  A A L  T Y Y  Y R M K  M P E  T A R
 721 tacaccgccc tgctgcaag aaacggaaag caggcagctg cagacatgtc taaggtagga
     Y T A L  V A R  N G K  Q A A A  D M S  K
 781 acaaagatga gttcttcctc tggccataga acaaatccat agtttattgg agaattataa

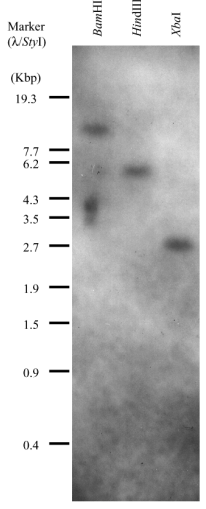
 841 gtatgataac caagactata atctgatact attatgatgg gcttcacaga attctttttg

 901 aacttcgtct tccttttga gtcacttgac atagttgagg tttttctatc aaattatttg

 961 gtcttgcatt cttgtcatca ctttcttgat tgagtggcaa tttcatgtaa ttataatgaa

1021 acgctcaaca ggtgttgcaa gtggatattg aatcagaaca agagaaggtg gagaaattta
           V L Q  V D I E  S E Q  E K V  E K F T
1081 cgcaagatcc cgcgaacagc tatggccttt tctcgaagga attcgcccgc cgccacggcc
     Q D P  R N S  Y G L F  S K E  F A R  R H G L
1141 tccacctcgt ggggacagcc accacgtggt tcttgctcga catcgctttc tatagccaaa
     H L V  G T A  T T W F  L L D  I A F  Y S Q N
1201 acctcttcca aaaggatatt ttcaccgcga tcgggtggct cccagcgggc aagaagatga
     L F Q  K D I  F T A I  G W L  P A A  K K M N
1261 acgccattca cgaggtatac aaaattgcta gggcacaaac cctgatcgct ctttgcagta
     A I H  E V Y  K I A R  A Q T  L I A  L C S T
1321 ctgtgcctgg gtattggttc actgtcgcga cgatcgatta catcggaaga ttcttcatcc
     V P G  Y W F  T V A T  I D Y  I G R  F F I Q
1381 aaatgatggg tttcgccatg atgtcaatct tcatgtttgc catcgccatc ccatacaacc
     M M G  F A M  M S I F  M F A  I A I  P Y N H
1441 actggaaaca tcaccatata ggattggtcg tgatgtactc actcacottc ttcttcgcca
     W K H  H H I  G F V V  M Y S  L T F  F F A N
1501 acttcgggcc aaacgcaaca acattcattg ttccgcgtga gatcttccgg gcgcggttga
     F G P  N A T  T F I V  P A E  I F P  A R L R
1561 gatccacatg ccatggcata tcggcggctg cagggaaagc tggagccatt gttggggctt
     S T C  H G I  S A A A  G K A  G A I  V G A F
1621 ttgggttctt gtatgcggcg caggataaaa caagccccga tgcaggttat aagccgggta
     G F L  Y A A  Q D K T  S P D  A G Y  K P G I
1681 tcggcgtgaa gaatgcattg cttgtgcttg ggggcattaa tcttgcggga atgttgttca
     G V K  N A L  L V L G  G I N  L A G  M L F T
1741 ctttgctcgt gccggagcca aagggcaggt cactggagga aattggagga gagaacatgg
     L L V  P E P  K G R S  L E E  I G G  E N M D
1801 atgataatga gatgggtgaa ggtagggaga tgcagcctcc ttcattggga ccatttggtg
     D N E  M G E  G R E M  Q P P  S L G  P F G G
1861 gttag
```

B



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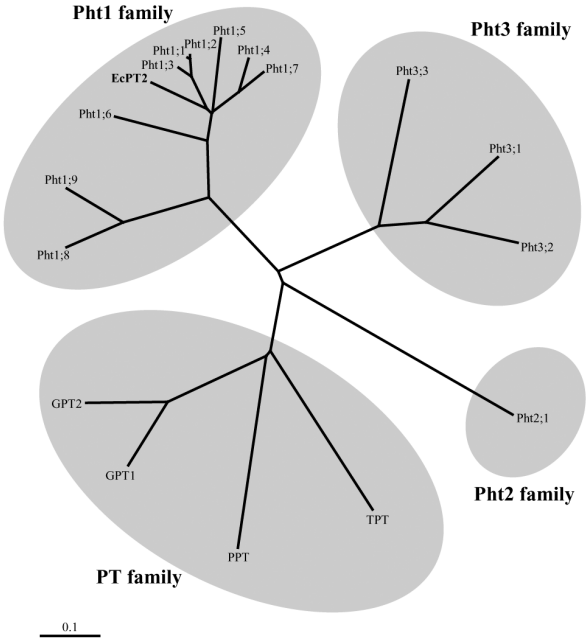

D

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                                                    TM1
EcPT2      1 MAKENLG-VLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFGVSLVTKLLGRIYYYEGK-
Pht1;1     1 MAEQQLG-VLKALDVAKTQLYHFTAIVIAGMGFFTDAYDLFGVSLVTKLLGRIYYFNPES
Pht1;4     1 MARGQLQ-VLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFGISLVTKLLGRIYYHWEGA
Pht1;6     1 MANEEQGSTLKALDVAKTQWYHVTAVVVSGMGFFTDSYDLFVISLITKLLGRIYYQVPGS

                                  TM2                                 TM3
EcPT2     59 DKPGSLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKRVYGLTLLIMIICSVGSGLSF
Pht1;1    60 AKPGSLPPHVAAAVNGVALCGTLSGQLFFGWLGDKLGRKKVYGLTLVMMILCSVASGLSF
Pht1;4    60 QKPGILPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKKVYGMTLMVMVLCSIASGLSF
Pht1;6    61 SSPGSLPDGISAAVSGVAFAGTFIGQIFFGGLGDKLGRKRVYGLTLLIMIICSICSGLSL

                                                    TM4
EcPT2    119 GDSFWSVIATLCFFRFWLGFGIGGDYPLSATIVSEYANKKTRGAFIAAVFAMQGFGILGG
Pht1;1   120 GHEAKGVMITLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGILAG
Pht1;4   120 GHEPKAVMATLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFVSAVFAMQGFGIMAG
Pht1;6   121 GRDPKTVMVTLCFFRFWLGFGIGGDYPLSATIMSEYSNKRTRGAFTAAVFGMQGIGILAA

                TM5                                 TM6
EcPT2    179 GIVALIVSSAFDHKFKAPFVEVNPVGSTVPQADYVWRIIVMLGALPAALTYYYRMKMPET
Pht1;1   180 GFVALAVSSIFDKKFPAPIYAVNRALSTPPQVDYIWRIIVMFGALPAALTYYWRMKMPET
Pht1;4   180 GIFALIISSAFEAKFPSPAYADDALGSTIPQADLVWRIILMAGALPAAITYYSRSKMPET
Pht1;6   181 GAVSLLVSAVFESKFPSRAYTLDGAASTVPQADYVWRIILMVGALPALLTYYWRMKMPET
                                                                        *

EcPT2    239 ARYTALVARNGKQAAADMSKVLQVDIESEQEKVESKFTQDPRNSYGLFSKEFARRHGLHLV
Pht1;1   240 ARYTALVAKNIKQAIADMSKVLQIDIELEERVEDDVKDPKQNYGLFSKEFLRRHGLHLL
Pht1;4   240 ARYTALVAKDAKQAASDMSKVLQVEIEPEQQKLEEISKEKSKAFGLFSKEFMSRHGLHLL
Pht1;6   241 ARYTALVSKNAEQAALDMIKVLNVDIEASAAKNDQ-ARVSDEFGLFSNKFLRRHGLHLL
             **

                TM7
EcPT2    299 GIATIWFLLDIAFYSQNLFQKDIFTAIGVLPAAKKMNAIHEVYKIARAQTLIALCSTVPG
Pht1;1   299 GTITSTWFLLDIAFYSQNLFQKDIFSAIGVIPKAAIMNAIHEVFRIARAQTLIALCSTVPG
Pht1;4   300 GTITSTWFLLDIAFYSQNLFQKDIFSAIGVIPPAQSMNATQEVFKIARAQTLIALCSTVPG
Pht1;6   300 GTASTWFLLDIAFYSQNLFQKDIFTTIGVLPSAKTMNAIQELYMIAKAQTIIACCSTVPG

                TM8                                 TM9
EcPT2    359 YWFTVAFIDVIGRFFIQNMGFAMMSIFMFAIATPYNHWK--HHIIGFVVMYSLTFFFANF
Pht1;1   359 YWFTVAFIDVIGRFKIQLNGFFMMTVFMFAIAFPYNHWIKPENRIGFVVMYSLTFFFANF
Pht1;4   360 YWFTVAFIDVIGRFAIQNMGFFMMTVFMFALATPYNHWTHKENRIGFVIMYSLTFFFANF
Pht1;6   360 YFFTVGFIDYMGRKKIQIMGFAMMTIFMLSLAIPYHHWTLPANRIGFVVLYSFTFFFSNF

                TM10                                TM11
EcPT2    417 GPNATTFIVPAEIFPARLRSICHGISAAAGKAGAIVGAFGFLYAAQ--DKTSPDAGYKPG
Pht1;1   419 GPNATTFIVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSQDKAKVDAGYPPG
Pht1;4   420 GPNATTFWVPAEIFPARFRSTCHGISAASGKLGAMVGAFGFLYLAQNPDKDKTDAGYPPG
Pht1;6   420 GPNATTFTVPAEIFPARIRSTCHGISAASGKAGAMVGSFGFSALVK-------------A
                ++++

                                    TM12
EcPT2    475 IGVKNALLVLGGINLAGMLFTLLVPEPKGRSLEELGGENMDDNEMGEGREMQPPSLGPFG
Pht1;1   479 IGVKNSLIMLGVLNFIGMLFTFLVPEPKGKSLEELSG----EAEVSHDEK-----------
Pht1;4   480 IGVRNSLIVLGVVNFIGILFTFLVPESKGKSLEEMSGEN-EDNENSNNDSRTVPIV-----
Pht1;6   467 LGMSNTLYIMAGINLLGLLFTFIPETNGKSLEELSG----ETEPEKIKEKIVV---------
                                                  ####

EcPT2    535 G
Pht1;1       -
Pht1;4       -
Pht1;6       -
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Acknowledgements

This work was supported by Research Institute of Innovative Technology for the Earth (RITE), the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Agriculture, Forestry and Fisheries of Japan.

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